

## Research Note

# Production and Stability of Patulin, Ochratoxin A, Citrinin, and Cyclopiazonic Acid on Dry Cured Ham

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MS 04-498: Received 29 October 2004/Accepted 6 March 2005

### ABSTRACT

Toxinogenic fungal species can be isolated from dry cured meat products, raising the problem of the direct contamination of these foods by mycotoxins known to be carcinogenic or potent carcinogens. Because the contamination of a food by mycotoxins can be considered a balance between production and degradation, the stability of mycotoxins on dry cured meat was also investigated. This study focused on patulin, ochratoxin A, citrinin, and cyclopiazonic acid that can be produced by fungal species previously isolated from dry cured meat products sold on the French market. We demonstrated that neither patulin nor ochratoxin A was produced on dry meat by toxigenic strains, whereas relatively high amounts of citrinin and cyclopiazonic acid were found after a 16-day incubation period at 20°C (87 and 50 mg/kg, respectively). After direct contamination, the initial content of patulin rapidly decreased to become undetectable after only 6 h of incubation at 20°C. For both citrinin and ochratoxin A, the kinetics of decrease at 20°C was less rapid, and the two toxins presented half-lives of 6 and 120 h, respectively. By contrast, more than 80% of the initial contamination in cyclopiazonic acid was still found on ham after a 192-h incubation period. Toxin stability was not affected by storage at 4°C. These results suggest that growth of toxigenic strains of *Penicillium* has to be avoided on dry meat products.

Several studies have shown that mold species belonging to the genera *Penicillium* and *Aspergillus* could be isolated from cured meat products such as ripened sausages or dry cured ham (1, 24). Interestingly, some of these strains are able to produce aflatoxins (11, 35), patulin (28), ochratoxins (12), citrinin (1, 10), or cyclopiazonic acid (27, 41) on culture medium. Also, aflatoxin B<sub>1</sub> (34) and ochratoxin A (8, 18) have been isolated from cured meat products, and the consumption of these products has been suspected to play a role in human exposure to mycotoxins (19, 36, 38, 42). However, although the toxigenic potential of molds is greatly linked to the substrate on which these molds grow, there are only a few studies designed to test the toxigenic potential of *Penicillium* or *Aspergillus* on cured meat products (7, 12, 44). Indeed, most of the studies demonstrating the toxigenic potential of molds isolated from dry cured meat were conducted on culture medium (27, 30, 45).

On the other hand, the occurrence of mycotoxins in food can be considered a balance between production and degradation. Therefore, investigation into the stability of mycotoxins on dry cured meat is of great interest because these foods can be stored for long periods without particular care. Unfortunately, no data concerning the stability of aflatoxins, patulin, ochratoxin A, citrinin, or cyclopiazonic acid were available on cured meat, whereas many studies were conducted to determine mycotoxin stability during

food processing on numerous foods, including cheese (4, 13, 26, 32, 40).

The aim of this work was to study the production of patulin, ochratoxin A, citrinin, and cyclopiazonic acid on dry cured meat product. The stability of these toxins at different temperatures of storage was also investigated. Patulin, ochratoxin A, citrinin, and cyclopiazonic acid were selected because they can be produced by fungal species previously isolated from dry cured meat products sold on the French market.

### MATERIALS AND METHODS

**Solvents and reagents.** All solvents and reagents were purchased from VWR International (Fontenay sous bois, France) and were of analytical grade. Mycotoxin standards (patulin, citrinin, cyclopiazonic acid, and ochratoxin A) were purchased from Sigma (Saint-Quentin Fallavier, France). Patulin was dissolved in ethyl acetate, ochratoxin A in 95° ethanol, citrinin in chloroform, and cyclopiazonic acid in methanol to obtain 1 mg/ml stock solutions that were stored at -20°C.

**Fungal growth and mycotoxin production.** Fungal strains *Penicillium patulum*, *Aspergillus ochraceus*, *Penicillium citrinum*, and *Penicillium viridicatum* produced patulin, ochratoxin A (OTA), citrinin, and cyclopiazonic acid (CPA), respectively. The strain of *P. patulum* was a generous gift from Dr. O. Puel (INRA, Pharmacology and Toxicology Unit, Toulouse, France), and *P. citrinum* was purchased at the Agricultural Research Service Culture Collection (Peoria, Ill.) and identified as NRRL 1841. The two other strains belong to our strain collection. They were isolated from dry cured meat or Vietnamese rice, and their toxigenic potential has already been characterized (41, 43).

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Strains were grown on malt agar medium (2% malt, 2% agar) supplemented with chloramphenicol (50 mg/liter) for 7 days at 20°C. From these fungal cultures, spore suspensions were prepared by the addition of 9 ml of Tween 80 (0.05%) to the petri dishes. Quantification of the number (CFU per milliliter) of these suspensions was then determined by the Petrifilm system as recommended by the manufacturer (3M, Malakoff, France). Spore suspension concentration was then adjusted to 10<sup>6</sup> CFU/ml.

Dry cured ham, produced in Spain or southwest France, was purchased in the French commercial market. Ten-gram pieces (5 by 5 by 0.5 cm) were placed in small incubation chambers (200 ml) containing 30 ml water at the bottom to avoid further drying of the meat. Meat pieces were not in direct contact with water. After this storage procedure, both aspect and odor of controls remained unchanged. Fungal contamination was done by incubating 100 µl of a 10<sup>6</sup> CFU/ml spore suspension on cured meat or yeast extract medium (YES; 2% yeast extract, 16% sucrose; Oxoid, Dardilly, France) several times at 20 and 4°C. All experiments were done three times, each in triplicate.

**Mycotoxin stability.** The stability of patulin, ochratoxin A, citrinin, and cyclopiazonic acid on dry cured meat samples was tested by direct contamination with 100 µl of a solution containing 50 µg of pure mycotoxin. Patulin was dissolved in ethyl acetate, OTA in ethanol, citrinin in chloroform, and CPA in methanol. Contaminated cured ham samples were then incubated several times at both 20 and 4°C in incubation chambers.

**Mycotoxin quantification.** Toxins were extracted from both culture media and meat samples by mechanical agitation and filtration through Whatman no. 1 filters. The toxins were separated by thin layer chromatography on silica gel 60 TLC plates (Merck, Nogent sur Marne, France). Mycotoxins were then quantified by fluorodensitometry with a Shimadzu CS930 fluorodensitometer (Shimadzu Corp., Kyoto, Japan). Quantification was by comparison with known amounts of standard spotted on the same plate. The procedures are briefly listed below.

(i) Patulin was extracted by ethyl acetate, separated by migration in toluene–ethyl acetate–formic acid (5/4/1, vol/vol/vol). Plates were revealed by spraying methylbenzothiazolinehydrazine 0.5% and heating 10 min at 130°C (5). The yellow spot of patulin was quantified at 430 nm. The limit of quantification was 100 µg/kg, and recovery of the method was 85%.

(ii) Ochratoxin A was extracted by acetonitrile–4% KCl<sub>aq</sub> (9/1, vol/vol), separated by migration in toluene–ethyl acetate–formic acid (5/4/1, vol/vol/vol), and quantified by fluorimetry at 333 nm (6). The limit of quantification was 20 µg/kg, and recovery of the method was 85%.

(iii) Citrinin was extracted by acetonitrile–4% KCl<sub>aq</sub> (9/1, vol/vol) and acidified by H<sub>2</sub>SO<sub>4</sub> to pH 3. The development system was toluene–ethyl acetate–formic acid (6/3/1, vol/vol/vol). Citrinin was then quantified by fluorimetric detection at 330 nm (4). The limit of quantification was 20 µg/kg, and recovery of the method was 95%.

(iv) Cyclopiazonic acid was quantified as already described by Landsen (20). Culture medium was extracted by methanol–chloroform (1/1, vol/vol). After filtration, extracts were partitioned against chloroform and filtered on phase separator filters. The development system was ethyl acetate–isopropanol–ammoniac (20/15/10, vol/vol/vol). After a drying step, cyclopiazonic acid was revealed on plates by spraying Ehrlich reagent. Quantification was at 600 nm. The limit of quantification was 200 µg/kg, and recovery of the method was 94%.

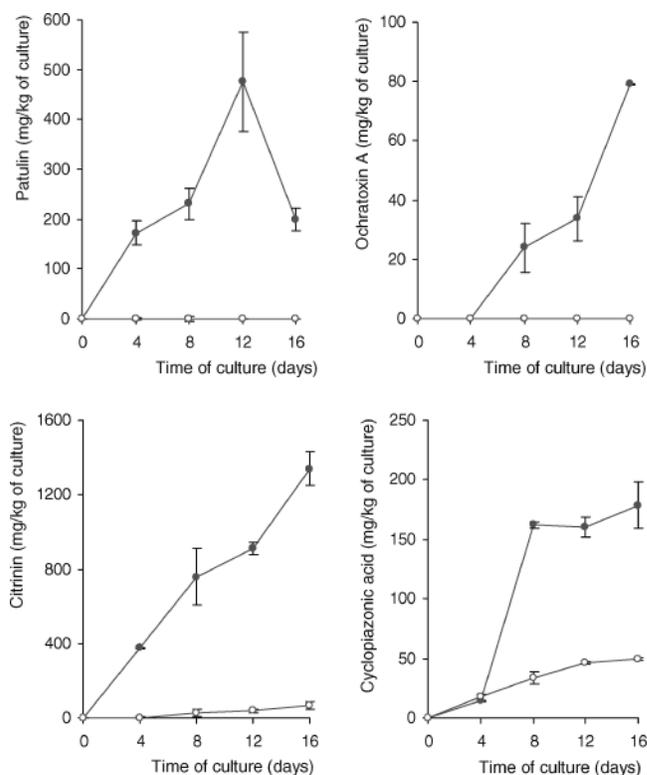


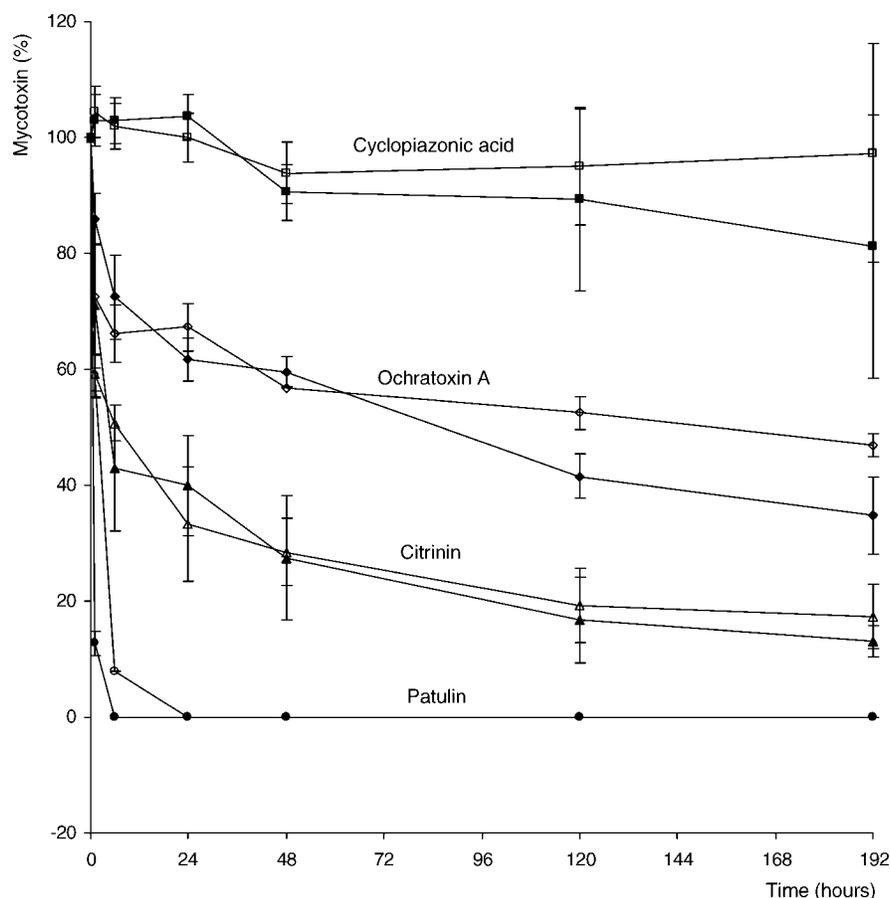
FIGURE 1. Production of patulin, ochratoxin A, citrinin, and cyclopiazonic acid on YES medium (light symbols) and on dry cured ham slices (bold symbols) at 20°C. Results are presented as the mean of three distinct experiments, each done in triplicate.

## RESULTS

**Mycotoxin production.** The toxigenic potential of selected mold strains was evaluated on both dry cured ham and YES. The results obtained in the different tested conditions are reported in Figure 1. Neither patulin nor ochratoxin A production was found on dry cured ham slices, whatever the incubation time tested. Nevertheless, after a 16-day incubation period, the strains of *P. patulum* and *A. ochraceus* used in this study were able to produce toxins on YES medium at about 475 and 79 mg/kg for patulin and ochratoxin A, respectively. Citrinin production by *P. citrinum* was rapidly observed after only 2 days of incubation at 20°C on YES medium and 4 days on meat samples. Levels as high as 86.9 mg/kg were obtained after 16 days of culture on dry cured ham, whereas the mycotoxin level reached 1,330 mg/kg on YES medium. Cyclopiazonic acid production was also observed after contamination of dry cured ham by toxigenic *P. viridicatum* spore suspension. Kinetics of production was comparable to that observed for citrinin, and about 50 mg/kg was obtained on dry cured ham after 16 days. About 180 mg/kg was obtained on YES medium. For the four tested toxins, no production was observed when meat slices or YES medium were incubated at 4°C (data not shown), even if a mild mycelium development could be observed after 10 days of incubation.

**Stability of mycotoxins.** The stability of pure mycotoxins added to meat slices of dry cured meat was tested at both 20 and 4°C (Fig. 2). At 20°C, the initial content of

FIGURE 2. Stability of patulin, ochratoxin A, citrinin, and cyclopiazonic acid at 20°C (bold symbols) and 4°C (light symbols) after contamination of dry cured ham slices with 50 µg of pure mycotoxins. Results are presented as the mean of three distinct experiments, each done in triplicate.



patulin decreased rapidly because 90% is lost after only 1 h of incubation. Patulin level became undetectable after 6 h of incubation. For citrinin, more than 50% of the initial content was lost after only 6 h of incubation at 20°C. Less than 15% of the toxin remained after 192 h of incubation. Kinetics of disappearance of OTA was slightly different because the half-life of the toxin was about 120 h, and about 30% of the initial contamination was still present after a 192-h incubation period at 20°C. By contrast, more than 80% of the initial content of CPA was still observed after 192 h of storage at 20°C. As shown in Figure 2, stability of the toxins was not strongly affected by storage at 4°C.

## DISCUSSION

Several studies have shown that cured meat products can be contaminated by fungal species belonging to the genera *Aspergillus* and *Penicillium* (1, 11, 27, 28, 30, 35, 45). Because some of these species are known to be toxigenic, they can contaminate this kind of product by compounds that have been identified as carcinogenic (aflatoxin B<sub>1</sub>), potent carcinogenic (OTA and citrinin), or mutagenic (CPA) (2, 3, 9, 14–17, 39). Recently, we showed that ripened cured meat products commercially sold in France could be contaminated by toxigenic *Penicillium* strains but not by *Aspergillus* species, some isolates producing patulin, OTA, citrinin, and CPA (40, 41). By contrast, only a few studies have demonstrated the presence of mycotoxins in cured meat products (8, 18, 34). This can be linked to the

lack of production of mycotoxins on this kind of substrate, to the rapid degradation of the toxins, or to both. That is why we studied both production and stability of different mycotoxins on dry cured ham.

In this study, the strain of *P. patulum* used was unable to produce any detectable amount of patulin in dry meat, whereas it produced around 475 mg/kg of this mycotoxin in YES culture medium. This result confirms that the toxigenic potential of molds is directly dependent on the substrate on which they are grown (22, 29, 30), in agreement with previous studies done on other substrates such as cheese (40), and can be explained by the high protein content of these kinds of foods. Indeed, it is now well demonstrated that the ability of patulin to bind with sulfhydryl compounds leads to rapid disappearance of the toxin (25). Because the mycotoxin cannot be recovered from these adducts (25), the contamination of meat products by patulin can be considered of little importance to consumer health.

In the same way, the *A. ochraceus* strain used was unable to produce detectable amounts of OTA on dry cured ham, whereas it produced 79 mg/kg of the toxin when cultured on YES medium. This result is in agreement with a previous study that found a highly toxigenic strain of *A. ochraceus* that produced only 0.4 to 6 mg/kg of OTA after 21 days of incubation on ham, whereas it was able to produce 151 mg/kg on rice culture (12). These different results obtained on ham and rice or YES medium can be explained by the low toxigenic potential of *Aspergillus* on this kind of substrate, the rapid degradation of the toxin, or both.

Here, we demonstrated that the half-life of OTA is about 120 h. This last result suggests that OTA might be converted into another molecule on dry cured ham. The toxicological signification of this conversion has to be elucidated.

By contrast, the toxigenic *Penicillium* strain was able to produce citrinin on dry cured ham. About 73 mg/kg has been observed after 16 days of incubation. Previous studies showed that citrinin-producing strains could be isolated from ripened meat products and cheeses (44, 45). However, no data are available concerning citrinin content in meat products, despite this toxin's suspected role in Balkan endemic nephropathy (31) and its mutagenicity (37). The study of the stability of citrinin at 20 and 4°C demonstrates that the half-life of the toxin is about 6 h, suggesting that citrinin is only partially stable on dry cured ham. This result agrees with those obtained on some cheeses and cereals (4, 13).

Two recent studies showed that *Penicillium* strains isolated from dry meat products could produce cyclopiazonic acid in culture medium (27, 41), but no survey is available on the contamination of dry cured meat products by CPA, despite its suspected role in "kodua poisoning" in humans (2, 33) and its mutagenicity on *Salmonella* Typhi assay. In this study, we demonstrated that the toxigenic strain of *P. viridicatum* used is able to produce cyclopiazonic acid on dry cured meat. We also demonstrated that cyclopiazonic acid is stable on dry meat products because more than 80% of the initial contamination is still recoverable after 8 days of incubation. Taken together, these results suggest that an accumulation of a relatively high level of CPA can be observed after contamination of dry cured meat. They suggest that fungal strains used in cured meat processing should be tested for their ability to produce CPA before use on commercial products. This recommendation is in agreement with a previous one concerning the use of fungal starters in cheese (21, 23).

This study shows that strains of *P. citrinum* and *P. viridicatum* can produce citrinin and cyclopiazonic acid, respectively, on dry cured meat products at relatively high levels. Some of these toxins, such as CPA, are quite stable on such a substrate, whereas others, such as patulin, rapidly disappear. The stability of citrinin and OTA can be considered as intermediate. Taken together, these results suggest that growth of toxigenic strains of *Penicillium* has to be avoided on dry meat products. This objective could be achieved by regular controls of fungal mycoflora that develop during the ripening period.

#### ACKNOWLEDGMENTS

The authors thank Sylviane Bailly and Pierrette Le Bars for helpful advice on mycological aspects and Mrs. Marie-Rose Trumel for technical assistance.

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